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Homeotic evidence for the appendicular origin of the labrum in *Tribolium castaneum*

Received: 8 June 2000 / Accepted: 14 November 2000 / Published online: 8 February 2001
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Abstract The ontogeny of the insect labrum, or upper lip, has been debated for nearly a century. Recent molecular data suggest a segmental appendage origin of this structure. Here we report the first arthropod mutation associated with a homeotic transformation of the labrum. *Antennagalea-5* (*Ag⁵*) transforms both antennal and labral structures to resemble those of gnathal appendages in *Tribolium castaneum*. This labral transformation suggests that the labrum is a fused structure composed of two pairs of appendage endites, and is serially homologous to the gnathal appendages.

Keywords Insect head · Homeotic transformation · Mandible · Labrum · Appendage

Introduction

The insect head capsule is a fused, composite structure with segmental origins that have been difficult to trace. Three gnathal segments – mandibular, maxillary, and labial – are identifiable in the embryo by their distinct appendages that are serially homologous to the thoracic appendages (Snodgrass 1960). The more anterior pregnathal segment number, identity, and order have long been a matter of debate (Rempel 1975). One widely held view is that the insect head is composed of a non-segmental anterior cap, the acron, which is thought to be the equivalent of the annelid prostomium, followed by six segments – labral, antennal, intercalary, mandibular, maxillary, and labial (Rempel 1975; Fig. 1A). Expression of the molecular markers *engrailed* (*en*) and *wingless* (*wg*)

confirms the segmental character of the antennal and intercalary segments, and suggests that the eye represents a segment rather than a feature of the acron (Schmidt-Ott and Technau 1992; Fig. 1B). The nature of the labrum remains controversial. It has been variously thought to be a feature of the acron, a simple cuticular structure of the first segment, the fused appendages of the first segment, or the fused appendages of the intercalary segment (Rempel 1975). Labral expression of the appendage marker gene *Distal-less* (*Dll*) in many arthropod classes, including Insecta, Crustacea, and Chelicerata (Popadic et al. 1998), suggests that the labrum is an appendage. In addition, the labrum responds in an appendage-like manner to reduced *Dll* levels in *Drosophila* by becoming greatly reduced (Cohen and Jørgensen 1989). This inference is corroborated by labral expression of *wg* (Schmidt-Ott and Technau 1992). However, the absence of *en* expression in the insect labrum, with *Drosophila* being one of

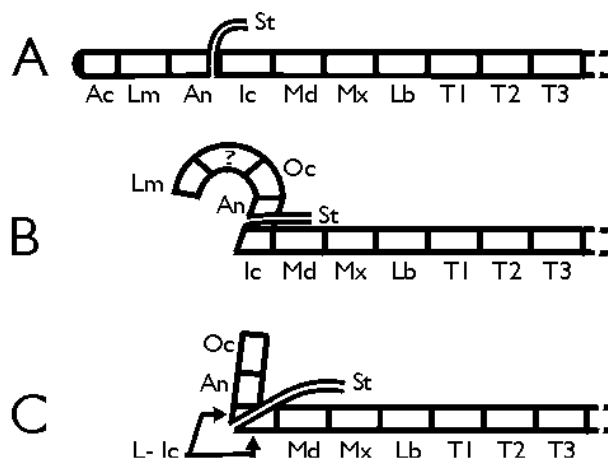


Fig. 1A–C Insect head segmental sequence. **A** Linear model (after Rempel 1975). **B** S-model (after Schmidt-Ott and Technau 1992). **C** Bent-Y model (this paper). ? Unknown, Ac acron, An antennal, Ic intercalary, L-Ic labro-intercalary, Lb labial, Md mandibular, Mx maxillary, Oc ocular, St stomodeum, T1–T3 thoracic segments 1–3

Edited by G. Jürgens

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the rare exceptions (Schmidt-Ott et al. 1994), casts doubt on its segmental derivation.

Homeotic mutations also can provide useful clues to segmental identity. For instance, the homeotic transformation of the insect antenna to thoracic leg in *antennapedia* (*ap*) mutants has helped establish the segmental appendage nature of the antenna. To our knowledge, homeotic transformations of the labrum have not previously been found either in *Drosophila* (Lindsley and Zimm 1992) or in any other arthropod. Herein, we describe a homeotic mutation affecting the labrum of a beetle, *Tribolium castaneum*, and revisit the question of the segmental derivation of this structure in the light of modern genetics and molecular biology.

Materials and methods

Reversion analysis

Beetles were reared at 30 °C on whole-wheat flour containing 5% (w/w) brewer's yeast. The *maxillopedic*^{Stumpy/Abdominal}*Miscadual*^{sclerotization1} (*mxp^{Stm}/A^{Mcs1}*) balanced stock (Beeman et al. 1989) was used for the reversion mutagenesis. One-week-old *mxp^{Stm}/A^{Mcs1}* adult males were treated with 4 kR of gamma irradiation (15-min exposure), then divided into five groups of 285 each (1,425 total) and held for 2 days at 30 °C for sperm maturation. To each of the five groups of males 195 two-week-old virgin *sooty* (*s*) females were added. Males were discarded after an additional 2 days and females were allowed to oviposit for several weeks. F1 adults were screened for reversion (loss) of either of the dominant mutations *mxp^{Stm}* (homeotic transformation of antennae to maxillary palps) or *A^{Mcs1}* (homeotic transformation of ventral abdominal segment 8 to segment 7). Revertants were recognized by their wild phenotype. In the absence of genetic reversion, all progeny would carry either the *mxp^{Stm}* or the *A^{Mcs1}* mutation. In addition to revertants, new dominant mutations were also detected. These mutants were crossed to a wild-type strain, *Georgia-1* (*Gal*), to remove incidental mutations. The F1 progeny bearing a new dominant mutation were then crossed to *Abdominal*^{Extra sclerite} (*A^{Es1}*) (Beeman et al. 1989) to establish balanced mutant stocks.

Specimen preparation

Specimens were prepared for laser confocal microscopy as follows: frozen or 70%-ethanol-preserved adult beetle heads were manually cleaned of debris or cleared in 1.0 N NaOH, rinsed, blotted dry on tissue paper, and mounted directly on double-sided sticky tape on a 75×25 mm glass microscope slide. Mouthparts and antennae were dissected from cleared or uncleared specimens and mounted in Euparal (ASCO Laboratories) on a 75×25 mm glass microscope slide.

A Zeiss LSM400 laser confocal microscopy system was used to document most specimens. Optical sections ranged from 40 to 570 μm and were recorded in the four line average mode with picture size of 512×512 pixels. Some specimens were documented using a Spot Camera II (Diagnostics) digital camera on an Olympus SZH10 Research Stereoscope or an Olympus BX60 compound microscope. Images were archived and digitally processed using Corel Photo-Paint 8, then arranged and labeled using CorelDRAW 8.

Results and discussion

This reversion mutagenesis generated the new dominant mutant, *Antennagalea-5* (*Ag⁵*) on the *mxp^{Stm}* chromo-

some. Both wild-type and mutant morphologies are described and interpreted below, and possible genetic control explored.

Ag⁵ genetics

The molecular lesions associated with *mxp^{Stm}* and *Ag⁵* are unknown. They appear to be tightly linked since they remain balanced without selection over *A^{Es}*, a reliable balancer of the HOM-C. Since *Ag⁵* is associated with gnathal transformation of anterior head structures, we performed complementation tests with *T. castaneum* *Deformed* (*TcDfd*) and *T. castaneum* *Cephalothorax* [*TcCx*; orthologs of *Drosophila* *Dfd* and *Drosophila* *Sex combs reduced* (*Scr*) respectively], two homeotic genes required for proper gnathal development. In these tests, *mxp^{Stm}Ag⁵/TcDfd* or *mxp^{Stm}Ag⁵/Cx⁶* adults were recovered at expected frequencies, indicating that these individuals are not deficient in *TcDfd* or *TcScr* activity. Curiously, cuticle preparations of additional progeny from these complementation tests revealed a class of embryos exhibiting the *TcDfd* null phenotype, but not the *Cx* null phenotype. This probable aneuploid class suggests that the *Ag⁵* mutation involves a complex chromosomal rearrangement that affects the *TcDfd* locus. We are pursuing a more detailed genetic and molecular analysis of this unusual allele.

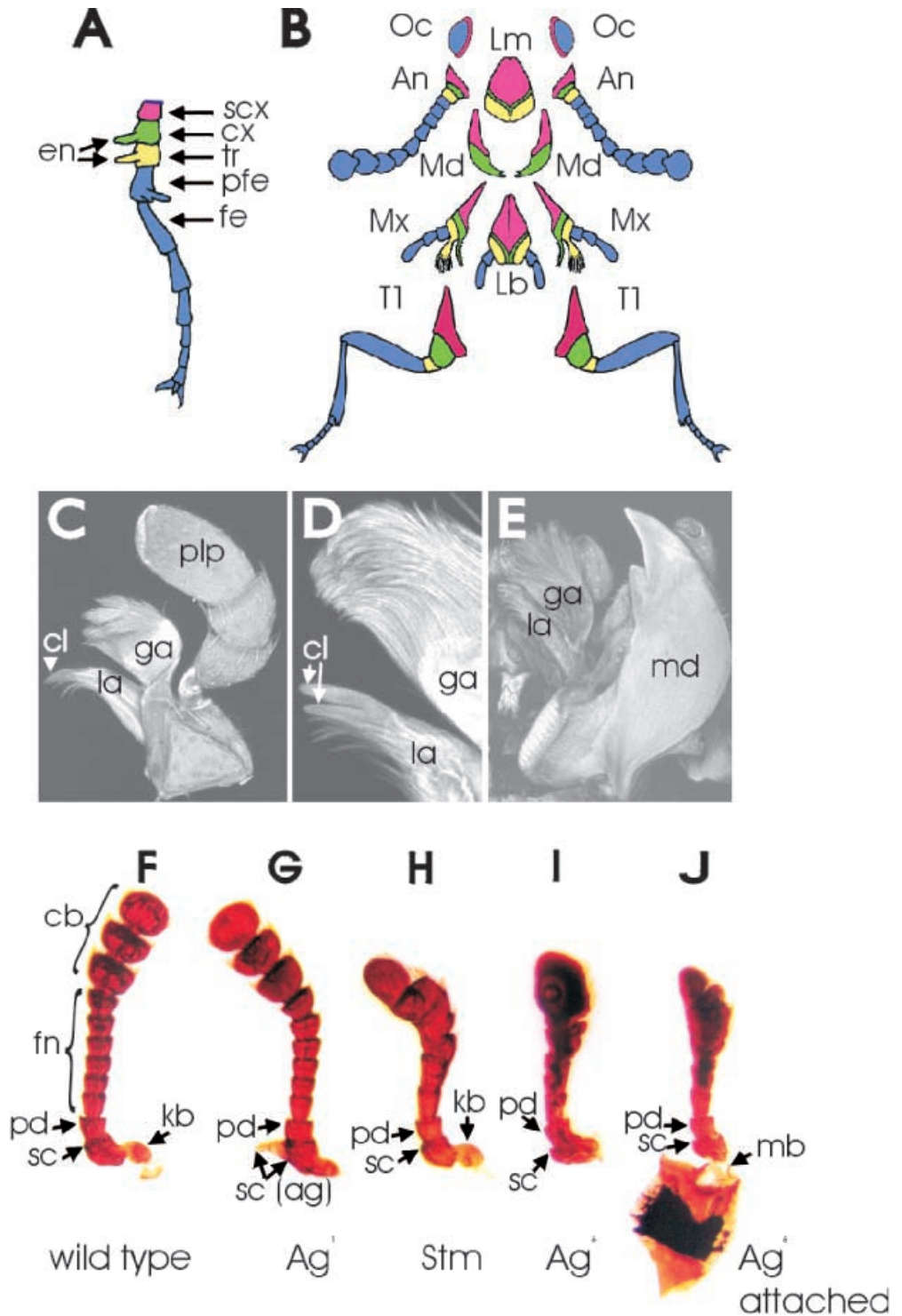
General insect appendage composition

Our interpretations of homeotic transformations are based upon an insect appendage segmentation model derived from Kukalová-Peck's insect fossil studies (1992). We have also incorporated studies of insect comparative anatomy and neurogenesis, detailed below.

The fossil evidence suggests that primitive insect appendages, including head appendages (Fig. 2A), were composed of a segmented basal region or "coxopodite", consisting of the subcoxa, coxa, and trochanter, and a segmented distal region or "telopodite," consisting of all segments distal to the trochanter. Medially projecting endites existed on the coxa and the trochanter. In the maxillary segment, the homologs of the trochanter and coxa are fused to form the stipes. The maxillary galea is the trochanteral endite and the lacinia is the coxal endite.

Most modern insect appendages have only some of these components (Fig. 2B) with the greatest number usually found on the maxillary and labial segments. In *Tribolium* adults, the maxillary appendages each bear two endites (galea and lacinia; Fig. 2B, Mx, yellow and green and Fig. 2C–E, "ga" and "la") and a telopodite (palp; Fig. 2B, Mx, blue, 3C, "plp" and Fig. 3, open arrows). The labial endites are fused medially and are unrecognizable as distinct structures (Fig. 2B, Lb). Thoracic segmental appendages (legs; Fig. 2B, T1) and the antennae (Fig. 2B, An) no longer bear endites. In contrast, the mandibular appendages (Fig. 2B, Md) no longer bear

Fig. 2A–J Insect appendages. Dorsal is up (**A,B**). Distal is up (**C–J**). **A** Ancestral head appendage (after Kukalová-Peck 1992). **B** Paired insect appendages. **C** Maxillary appendage. **D** Close-up of distal galea and lacinia. **E** Dorsal view of gnathal appendages, right side. **F** Wild-type antenna. **G** *Ag¹* antenna. **H** *mxp^{Stm}* antenna. **I** *Ag⁵* antenna. **J** *Ag⁵* antenna with partial head capsule attached. Red epicoxa (coxopodite), green coxa (coxopodite), yellow trochanter (coxopodite), blue palp, leg or antenna (telopodite), ag antennal galea, An antenna, cb club, cl claw; cx coxa, en endites, fe femur, fn funicle, ga maxillary galea, kb knob, la maxillary lacinia, Lb labium, Lm labrum, mb membranous base, md mandible, Mx maxilla, Oc ocular, pd pedicel, pfe prefemur, plp palp, sc scape, scx subcoxa, T1 first thoracic legs, tr trochanter



telopodites (palps), and the intercalary segment is thought to entirely lack appendages (not shown).

Appendage homologies

The mandible is homologous to the maxillary lacinia based on musculature (Snodgrass 1960). This researcher

showed that the mandible is composed of basal appendage elements equivalent to the maxillary cardo (precoxa – also referred to as the subcoxa) and stipes (coxa) with a greatly enlarged lacinial homolog (see Fig. 2E for mandible-lacinia size comparison). This homology is also corroborated by the work of Meier and Reichert (1991) on the neurogenesis of the grasshopper. Their work shows that the developing mandible and the maxillary la-

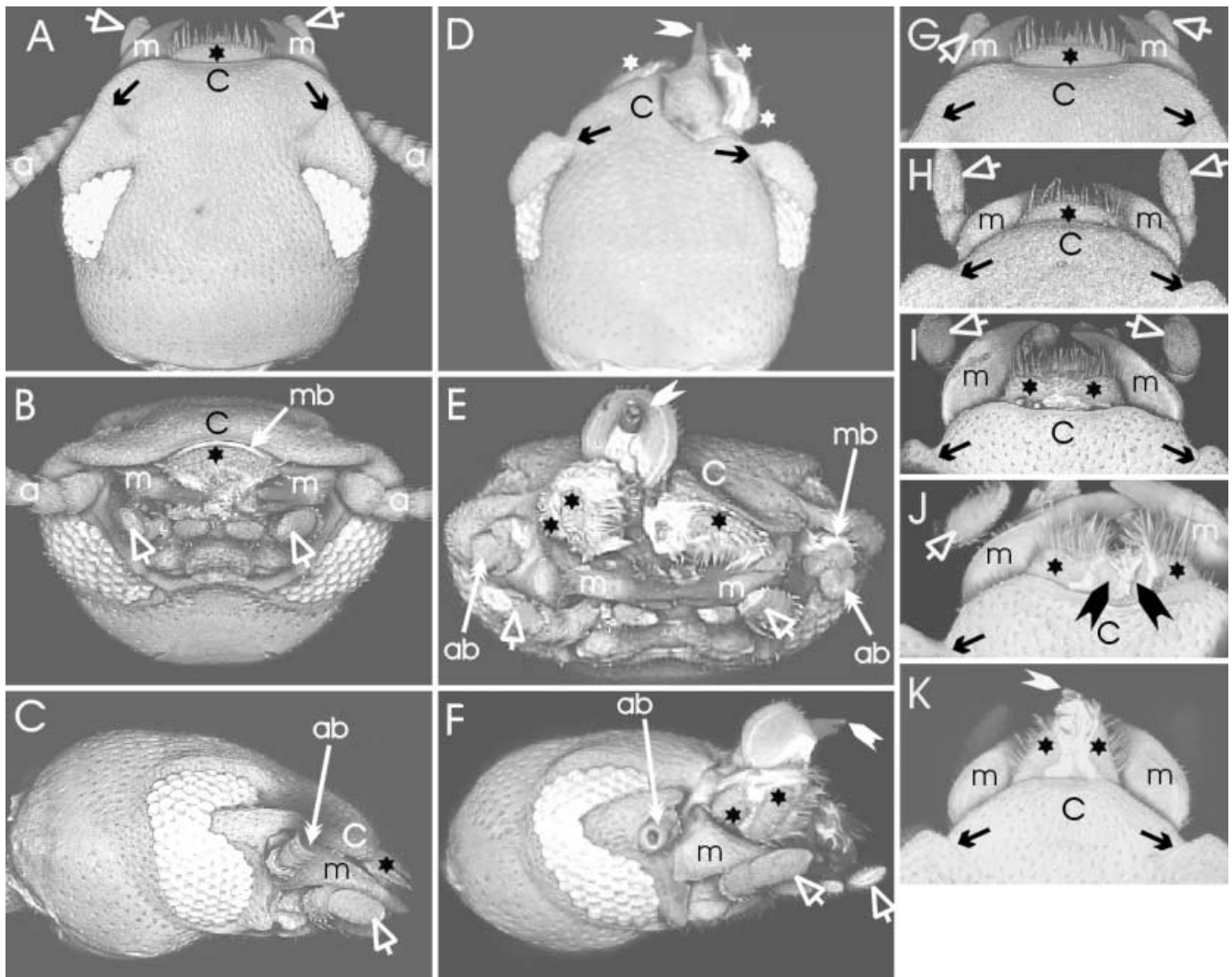


Fig. 3A–K *Tribolium castaneum* adult head morphology and labral transformations. Anterior is up (A,D,G–K). Dorsal is up (B,E). Anterior is to the right (C,F). A Wild-type dorsum. B Wild-type front. C Wild-type side. D *Antennagalea-5* (*Ag⁵*) dorsum. E *Ag⁵* front. F *Ag⁵* right side. G–K Anterior dorsal close-ups. G Wild-type. H Subtly transformed *Ag⁵* labrum. I–K Noticably transformed *Ag⁵* labra. * Labrum or labral-lateral plates, solid arrow heads labral medial plate or labral “tooth”, open arrow maxillary palp, small black arrows epicranial arms/sutures, a antenna, ab (with double barbed arrow) antennal base (antenna removed for clear view of structures), C clypeus, m mandible, mb (with double barbed arrow) membranous area

cinia share a neurogenic pattern not found in the maxillary galea or palp. Kukalová-Peck (1992) proposes that the mandible consists of a completely fused coxopodite, including the lacinia as molar and the galea as the incisor.

While musculature, neurogenesis, and paleontology support a lacinial homology for the mandible, gene expression of *Dll* is not readily consistent with this view. Mandibular expression of *Dll* is found in some crustaceans which bear mandibular palps, but is absent in insects (Popadic et al. 1998). The maxillary lacinia and homologous labial glossa do express *Dll* (Rogers and

Kaufman 1997). This brings into question either the mandible’s composition, or the consistency of *Dll* as a lacinial homolog marker. Perhaps genes such as *cap’n’collar* (*cnc*) or *buttonhead* (*btd*), which are not expressed in maxillary or labial segments, repress *Dll* expression in the mandible.

T. castaneum adult head morphology

The dorsal surface of the wild-type head capsule is gently convex, with its anterior rim relatively uninterrupted from eye to eye (Fig. 3A). The antennae, labrum, mandibles, and maxillary and labial palps project from beneath the anterior rim. The eyes, located on the sides of the head, are partially divided into dorsal and ventral compartments by the rim (Fig. 3C). Antennae consist of two basal segments (scape and pedicel), six smaller funicle segments, and a distal, three-segment club (Fig. 2F). Each antennal segment bears short setae at its distal margin (Fig. 3A,B). The scape is distally cylindrical and narrows towards the base, ending in a sclerotized lateral “knob” that articulates with the antennal socket of the

head capsule (Fig. 2F, “kb”). The small, flat labrum (star in Fig. 3A–C) bears several rows of setae along its distal margin. It is attached by membrane to the underside of the anterior head rim (“mb” in Fig. 3B) and is capable of limited movement. Ventral to the labrum lie the heavily sclerotized mandibles (“m” in Fig. 3A–K), with the maxillary appendages (open arrows in Fig. 3A–K) ventral to the mandibles and the smaller, basally-fused labial appendages nested between the maxillary appendages.

Ag⁵ larval head morphology

The wild-type larval head capsule lacks an anterior rim between the ocelli. Heterozygous *Ag⁵* larvae have normal appearing antennae, but approximately 10% have a split labrum, a probable reversion to a paired appendage (data not shown). *Ag⁵* homozygotes die early in embryogenesis and thus generate no informative phenotype.

Ag⁵ adult head morphology

Antennal morphology

The *mxp^{Stm}* mutation features a weak asymmetry of the antennal scape characterized by a slight dorsal enlargement at the distal end (Fig. 2H). *Ag⁵* was first identified by its enhancement of the *mxp^{Stm}* scape phenotype (Beeman et al. 1996). In *Ag⁵*, the distal scape dorsum is further enlarged and often bears a small, blunt setiferous projection (Fig. 2I,J). An even stronger scape phenotype has previously been described for the *Ag¹* mutation (Stuart et al. 1991; Fig. 2G) that was induced on a wild-type chromosome. The *Ag¹* and similar *Ag⁵* scape morphologies are interpreted as homeotic transformations of scape to maxillary galea, or antennal base to maxillary base.

Kukalová-Peck (1992) interprets the antennal scape as the subcoxal homolog and the pedicel as the coxal homolog. However, the appearance of a galea-like, rather than lacinia-like structure on the scape suggests that the scape may be the trochanter homolog instead. Coxal and subcoxal homologs have possibly fused with the head capsule.

In contrast to *mxp^{Stm}* and wild-type, the *Ag⁵* scape has a membranous base (Figs. 2J, “mb”, 3E, “mb”) rather than a sclerotized “knob”. This membranous base appears to alter the antennal articulation with the head capsule and might be responsible for the more ventral carriage of the *Ag⁵* antennae. The probable scape homolog, the maxillary stipes and galea, does not have an obviously equivalent membranous area. It is possible that a smaller membranous region on the stipes/galea may take on larger proportions when rendered in a partially transformed antennal base.

Labral morphology

Ag⁵ also causes a dominant modification of the anterior adult head capsule. In *Ag⁵* heterozygotes, the anterior head rim is always indented at the epicranial arms (black arrows in Fig. 3) due to the shortening of the clypeus (the portion of the anterior head between the epicranial arms; “C” in Fig. 3). The shortened clypeus exposes portions of the dorsal labrum, mandibles, and antennae normally covered by the anterior rim in the wild-type head.

A subtle modification of the labrum is also found in all adults examined thus far (>50). The dorsal sclerotized cuticle of the wild-type labrum appears continuous with the two arms of a more proximal V-shaped structure found on the oral side of the labrum (epipharynx). In *Ag⁵*, the dorsal labral cuticle is becoming membranous at its base, and the arms of the V-shaped structure are shortened and no longer continuous with the reduced distal labral cuticle (data not shown).

In addition to these subtle, invariant effects of *Ag⁵*, the normally small, flat adult labrum undergoes a more severe gross morphological transformation in around 2.7% of the beetles (30/1,111 *Ag⁵ mxp^{Stm}/A^{Es1}* examined as pupae). This modification ranges in expression from a midline longitudinal membranous strip and/or a slight elongation of the labrum (Fig. 3I,J) to a striking transformation of both the labrum and clypeus into enlarged, complex mandible-like structures that sometimes include a strongly sclerotized, tooth-like tip (Fig. 3D–F,K). When the labrum is expressed as a paired structure, two categories of sclerotized plates (lateral and medial) can be present separated by membrane, and each with a characteristic morphology. The lateral plates are often found without accompanying medial plates, but medial plates are not found in the absence of lateral plates. The medial plates (arrowheads, Fig. 3J,K), when expressed, are usually smaller than the lateral plates, and can appear as a single, or pair of small, irregular discs. When more strongly expressed, the medial plates tend to be elongated and sometimes bear a tooth-like tip (in about 20% of the transformations). The lateral plates (stars, Fig. 3D–F,I–K) show less size and shape variation than the medial plates and, in their weakest recognizable expression (similar to Fig. 3I), appear to each represent one half of the wild-type labrum. They are occasionally subdivided into proximal and distal sclerites (stars, Fig. 3F).

Is the labrum an appendage?

The homeotically transformed labrum found in the *Ag⁵* mutant displays morphological characteristics consistent with a multiple appendage endite composition. We interpret the medial plates as lacinia homologs and the lateral plates as galea homologs. Thus, the labrum is a probable appendage base (coxopodite).

The following four features of the transformed, medial labral structure (Fig. 3D–F) strongly resemble the mandible:

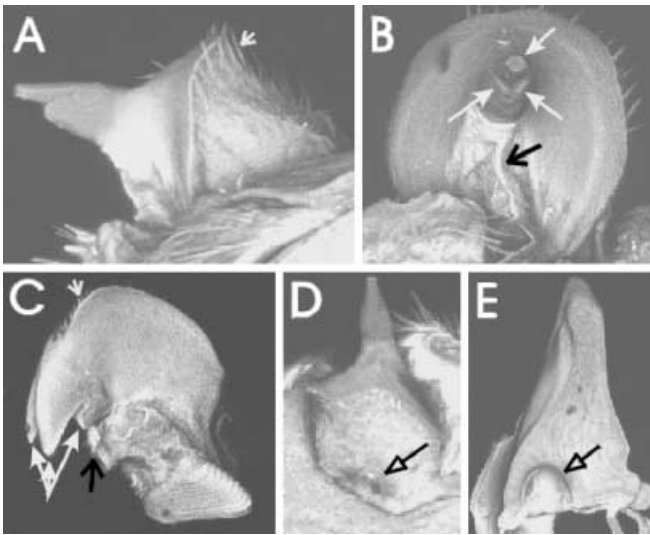


Fig. 4A–E Comparisons of *T. castaneum* adult mandible with transformed labrum. Dorsal is up (A,B). Lateral is up (C). Distal is up (D,E). **A** Left side of labral “tooth”. **B** Labral “tooth” anterior. **C** Right wild-type mandible, medial view. **D** Dorsal labral “tooth”. **E** Wild-type mandible (same relative view as D). Long white arrows incisor teeth, short white arrows setae, open arrows socket for articulation with head capsule, black arrows membranous prostheca

1. In wild-type *T. castaneum* each mandible always shows a characteristic “handedness.” While the left mandible has a bi-toothed incisor, the right mandible is tri-toothed distally (El-Kifl 1953; Fig. 4C). The transformed labral structure appears on the right side of the head and is tri-toothed (Fig. 4B).
2. At their dorso-lateral bases, wild-type mandibles bear a semicircular indentation that serves as a socket for the dorsal articulation with the head capsule (El-Kifl 1953; Fig. 4E). This feature is mimicked at the corresponding dorsal base of this specimen’s transformed labral structure (Fig. 4D).
3. The convex lateral surface of the wild-type mandible bears a number of small setae, while the distal biting surface has none (Fig. 4C), a pattern also duplicated on this mandible-like labral structure (Fig. 4A).
4. On the concave oral side, the wild-type mandible bears a flexible membranous ridge, the prostheca, between the incisor and the molar (Fig. 4C). A similar membranous area is found on the corresponding surface of the labral structure (Fig. 4B).

This labral “mandible” shows a dorsal rotation of almost 90 °C relative to the orientation of the wild-type mandible (Fig. 3D–F). This displacement might be caused by the encroachment of the lateral plate, a structure not present on the mandibular segment. Alternatively, it might reflect a common rotation of pregnathal appendages relative to the gnathal appendages. For example, the antennal “galea” of all *Ag* mutants shows a similar dorsal rather than medial orientation. Thus, this rotation might be caused by the migration of these anterior segments around the stomodeum during development.

Two features of the lateral plates of the transformed labrum resemble enlarged versions of the maxillary galea. The elongating distal setae of the lateral plates (Fig. 3I,J) resemble the long distal setae found on the maxillary galea (Fig. 2C–E). Additionally, as mentioned above, the maxillary galea consists of proximal and distal sclerotized plates with an intervening membranous region (El-Kifl 1953), a pattern also found in the more strongly transformed lateral plates of *Ag*⁵ (Fig. 3F).

Probable composition of the wild-type labrum

In weak to moderately transformed labra, large lateral plates (galeal homologs) separated by a membranous medial region are consistently present. In contrast, medial plates (mandibular/lacinal homologs) of variable size appear sporadically. We infer from this transformed labral phenotype that the wild-type labrum consists largely, if not entirely, of paired galeal components.

Palp-like structures have not been found in any *Ag*⁵-transformed labra examined thus far. The apparent absence of palps suggests a probable endite source of labral *Dll* expression in wild-type embryos. The labra of *Tribo- lium* (S.J. Brown, unpublished observation) and other insects (Panganiban et al. 1994; Popadic et al. 1998) express *Dll* during normal development. If labral *Dll* expression is under the same genetic control as maxillary and labial expression, then either a lacinal homolog (Rogers and Kaufman 1997), a galea homolog, or both (Niwa et al. 1997), would be the presumed source of labral expression, and therefore an implied part of the wild-type labrum. It is possible that unique genetic controls govern *Dll* expression in both the labrum and the mandible, and that lacinal *Dll* expression is repressed in these two segments. Labral *Dll* expression would then arise either from an undetected vestigial telopodite or from the galea homolog.

Does the labrum represent a segment?

The apparent appendicular nature of the labrum raises an interesting question. The labrum has often been assumed to be part of an acron. However, by definition, an acron is a structure that has no appendages, so the labrum must not be part of an acron. If an appendage always represents a segment, which segment does the labrum represent? We present evidence for the segmental identity of the labrum in a separate article in this journal issue (Haas et al. in press).

Acknowledgements All programs and services of the U.S. Department of Agriculture are offered on a nondiscriminatory basis, without regard to race, color, national origin, religion, sex, age, marital status, or handicap. This work was partially supported by National Institutes of Health grant HD29594 and National Science Foundation grant MCB963–0179.

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